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Application of CRISPR/Cas9 Technology in Editing Poplar Drought Resistance Genes

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Abstract Poplars (genus *Populus*), as fast-growing deciduous trees, hold significant ecological and economic importance. They contribute to carbon sequestration, soil stabilization, and provide habitats for wildlife, while also being widely used in timber, paper production, and bioenergy. However, drought stress poses a major challenge to the growth, productivity, and survival of poplars. The advent of CRISPR/Cas9 technology has revolutionized plant genetic engineering, offering precise and efficient genome editing capabilities. This study systematically introduces the basic principles of CRISPR/Cas9 technology and its applications in plant science, particularly how editing key drought-resistance genes can enhance the drought tolerance of poplars. Additionally, we discuss practical applications of CRISPR/Cas9-mediated gene editing in poplars, as well as challenges such as off-target effects, regulatory and ethical considerations, and environmental and ecological impacts. In the future, continuous innovation in CRISPR/Cas9 technology and its combination with other biotechnological methods are expected to further improve the drought resistance of poplars, promoting their commercial application and large-scale deployment. This study not only provides new approaches for improving the drought resistance of poplars but also offers valuable references for the research and application of stress resistance in other forest species.
Keywords Poplar; CRISPR/Cas9; Drought resistance; Gene editing; Plant genetic engineering

1 Introduction

Poplars (genus *Populus*) are fast-growing deciduous trees that play a significant role in various ecosystems. They are widely distributed across the Northern Hemisphere and are known for their rapid growth and adaptability to different environmental conditions. Poplars are crucial for their ecological functions, including carbon sequestration, soil stabilization, and providing habitat for wildlife. Additionally, they are economically important for their use in timber, paper production, and as bioenergy crops (Fan et al., 2015).

Despite their adaptability, poplar trees are not immune to abiotic stresses, particularly drought. Drought stress poses a significant challenge to poplar cultivation, affecting growth, productivity, and survival. Drought conditions lead to reduced water availability, which can cause physiological and biochemical changes in poplars, ultimately impairing their growth and development. Addressing drought stress in poplars is essential for maintaining their ecological and economic benefits, especially in the face of climate change (Zafar et al., 2020).

CRISPR/Cas9, derived from the prokaryotic adaptive immune system, allows for targeted modifications of specific genes, making it a powerful tool for crop improvement (Arora and Narula, 2017; Borrelli et al., 2018; Ahmad et al., 2020). This technology has been successfully applied to enhance disease resistance, improve nutritional content, and develop abiotic stress tolerance in various plant species (Bortesi and Fischer, 2015; Chandrasekaran et al., 2016; Ma et al., 2016). In the context of poplar trees, CRISPR/Cas9 presents a promising approach to edit genes associated with drought resistance, thereby enhancing their resilience to water scarcity (Fan et al., 2015; Badhan et al., 2021).

This study aims to utilize CRISPR/Cas9 technology to edit drought resistance genes in poplar trees, exploring its potential to enhance poplars' adaptability to drought conditions. By precisely targeting and modifying drought-responsive genes, we hope to develop poplar varieties better suited to withstand drought conditions,

ensuring their sustainability and productivity in changing climates. This study not only provides new insights for improving drought resistance in poplars but also offers valuable references for the study of stress tolerance in other plants.

2 CRISPR/Cas9 Technology: Mechanism and Applications

2.1 Basics of CRISPR/Cas9 gene editing

CRISPR/Cas9, which stands for Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated protein 9, is a revolutionary gene-editing technology that allows for precise modifications at specific locations within the genome. The system comprises two key components: the Cas9 enzyme, which acts as molecular scissors to cut DNA, and a single guide RNA (sgRNA) that directs Cas9 to the target DNA sequence. This technology has been widely adopted due to its simplicity, efficiency, and versatility in various organisms, including plants (Badhan et al., 2021; Park et al., 2022).

2.2 CRISPR/Cas9 applications in plant science

CRISPR/Cas9 has been extensively utilized in plant science to enhance traits such as disease resistance, stress tolerance, and yield improvement. For instance, in chickpea, CRISPR/Cas9 was used to edit the *4CL* and *RVE7* genes, which are associated with drought tolerance, demonstrating high-efficiency editing and providing insights into drought stress mechanisms (Badhan et al., 2021). Similarly, in rice, the *OsSAP* gene was edited to study its role in drought stress, showing that CRISPR/Cas9 can significantly reduce breeding cycles and improve stress-related traits (Park et al., 2022). Additionally, CRISPR/Cas9 has been employed to develop virus-resistant cucumber plants by targeting the *eIF4E* gene, resulting in non-transgenic plants with enhanced resistance to multiple viruses (Chandrasekaran et al., 2016). In forest trees, CRISPR/Cas9 has been applied to develop new drought-resistant cultivars and regulate lignin biosynthesis, showcasing its potential in tree genetic studies and breeding (Chen and Lu, 2020).

2.3 Advantages of CRISPR/Cas9 over traditional breeding techniques

CRISPR/Cas9 offers several advantages over traditional breeding techniques. It allows for precise and targeted modifications without the need for extensive backcrossing, which is time-consuming and labor-intensive. For example, the development of virus-resistant cucumber plants using CRISPR/Cas9 did not require long-term backcrossing, unlike traditional methods (Chandrasekaran et al., 2016). CRISPR/Cas9 can significantly shorten breeding cycles, as demonstrated in rice, where the technology enabled rapid generation of drought-tolerant cultivars (Park et al., 2022). CRISPR/Cas9 can be used to edit multiple genes simultaneously, providing a more comprehensive approach to trait improvement. This was evident in the chickpea study, where both *4CL* and *RVE7* genes were edited to enhance drought tolerance (Badhan et al., 2021). Overall, CRISPR/Cas9 represents a powerful tool for plant breeders and researchers, offering unprecedented opportunities for crop and tree improvement (Chandrasekaran et al., 2016; Chen and Lu, 2020; Badhan et al., 2021; Park et al., 2022).

3 Drought Resistance in Poplar Trees

3.1 Physiological and molecular responses to drought stress

Poplar trees, like many other plant species, exhibit a range of physiological and molecular responses to drought stress. These responses are crucial for their survival and adaptation in water-limited environments. Physiologically, drought stress in poplar trees leads to reduced leaf water potential, stomatal closure, and decreased photosynthetic rates. These changes help to minimize water loss and maintain cellular turgor pressure. On a molecular level, drought stress triggers the expression of various stress-responsive genes, including those involved in the synthesis of osmoprotectants, antioxidants, and stress-related proteins (Figure 1). These molecular responses are part of a complex regulatory network that helps the plant to cope with and adapt to drought conditions (Arora and Narula, 2017; Zhu et al., 2020; Wang et al., 2022).

The study by Arora and Narula (2017) demonstrates the mechanism of CRISPR/Cas9 in modifying the poplar genome. The process begins with the acquisition phase, where foreign DNA is integrated into the CRISPR locus of the bacterial genome. This locus is then transcribed into a primary transcript and processed into crRNA with the help of tracrRNA during crRNA biogenesis. During the interference phase, the Cas9 endonuclease forms a

complex with crRNA and cleaves the foreign DNA near the PAM region. This targeted cleavage mechanism allows for precise genetic modifications in poplar, enabling researchers to knock out detrimental genes or insert beneficial ones to enhance drought tolerance. The use of CRISPR/Cas9 in poplar demonstrates its potential for improving genetic traits, contributing to the development of more resilient tree varieties capable of withstanding environmental stresses.

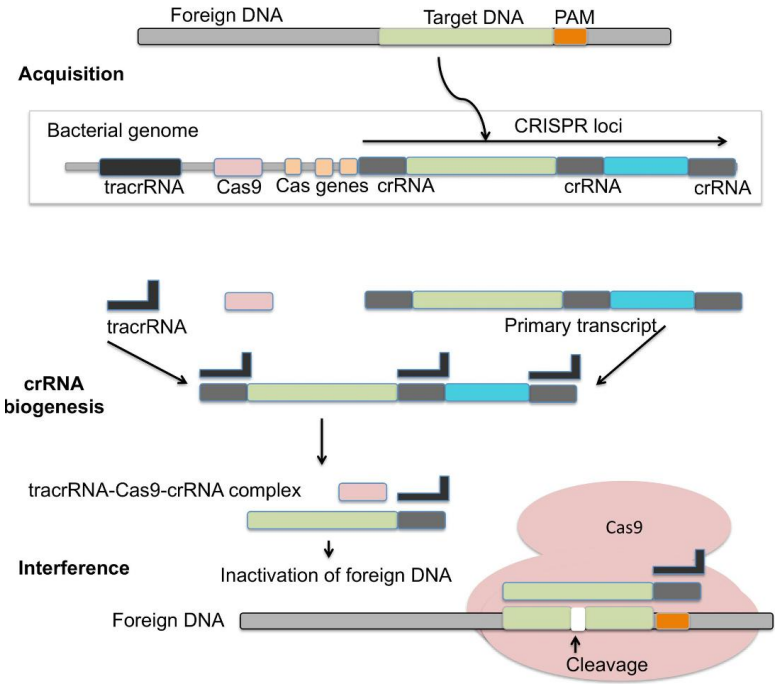

Figure 1 Mechanism of CRISPR/Cas9 action (Adopted from Arora and Narula, 2017)

3.2 Key drought resistance genes in poplar

Several key genes have been identified in poplar that play significant roles in conferring drought resistance. These include genes involved in the biosynthesis of abscisic acid (ABA), a hormone that regulates stomatal closure and other drought responses. Additionally, genes encoding for dehydrins, late embryogenesis abundant (LEA) proteins, and aquaporins are crucial for maintaining cellular hydration and protecting cellular structures during drought stress. The identification and functional characterization of these genes have been facilitated by advanced genomic and transcriptomic studies, which have provided insights into the complex genetic networks underlying drought resistance in poplar (Chen and Lu, 2020; Badhan et al., 2021; Park et al., 2022).

3.3 Traditional methods for enhancing drought resistance

Traditional methods for enhancing drought resistance in poplar trees have primarily relied on selective breeding and hybridization. These approaches involve selecting and cross-breeding individuals with desirable traits, such as deep root systems, efficient water use, and robust stress responses. While these methods have been successful to some extent, they are often time-consuming and labor-intensive. Moreover, the genetic basis of drought resistance is complex and involves multiple genes, making it challenging to achieve significant improvements through traditional breeding alone. Recent advancements in molecular biology and genetic engineering, such as CRISPR/Cas9 technology, offer new opportunities to enhance drought resistance in poplar more efficiently and precisely (Chandrasekaran et al., 2016; Arora and Narula, 2017; Borrelli et al., 2018; Chen et al., 2019).

4 Using CRISPR/Cas9 to Edit Drought-Resistance Genes in Poplar

4.1 Selection of target genes for drought resistance

The selection of target genes is a crucial step when applying CRISPR/Cas9 technology to enhance the drought resistance of poplar. Genes associated with drought resistance typically play roles in various physiological and biochemical pathways, such as lignin biosynthesis, reactive oxygen species (ROS) scavenging, and transcriptional regulation. For example, the 4-coumarate-CoA ligase (*4CL*) gene involved in the lignin biosynthesis pathway was

targeted in chickpeas to understand its role under drought stress (Badhan et al., 2021). Similarly, the MYB transcription factor Reveille 7 (RVE7), which regulates the circadian rhythm, was edited to study its effect on drought resistance (Badhan et al., 2021). These examples highlight the importance of selecting genes that play significant roles in the plant's response to drought conditions.

4.2 CRISPR/Cas9 vector construction and delivery methods

The construction of CRISPR/Cas9 vectors and their delivery into poplar cells are pivotal for successful gene editing. The CRISPR/Cas9 system typically involves the use of a Cas9 endonuclease and a single guide RNA (sgRNA) that directs the Cas9 to the specific genomic location. Various methods have been employed to deliver these components into plant cells, including Agrobacterium-mediated transformation, which has been successfully used in poplar (Fan et al., 2015). Additionally, the use of ribonucleoproteins (RNPs) composed of Cas9 and sgRNA has been shown to reduce off-target effects and avoid integrational mutagenesis (Arora and Narula, 2017; Mout et al., 2017). Nanoparticle-based delivery approaches are also emerging as efficient methods for direct cytosolic delivery of Cas9-RNP complexes, achieving high gene editing efficiencies (Mout et al., 2017).

4.3 Case studies of successful gene editing in poplar for drought resistance

Some studies have demonstrated the successful application of CRISPR/Cas9 technology in editing various traits in poplar trees, including drought resistance. For example, editing the phytoene desaturase gene (PDS) in an interspecific hybrid poplar (*Populus davidiana* × *P. bolleana*) achieved high mutation efficiency and generated albino phenotypes, indicating successful gene knockout (Wang et al., 2020). The research team focused on a hybrid poplar variety called Shanxin yang, known for its good cold and drought resistance. Through Agrobacterium-mediated transformation, recombinant plasmids containing the Cas9 expression cassette and two sgRNA cassettes were introduced into poplar leaves. The results showed that transgenic poplars with PDS gene mutations exhibited various phenotypes, including complete albinism, variegation, and light green phenotypes. The study demonstrates that the CRISPR/Cas9 system can efficiently edit the poplar genome, providing a new approach for improving drought resistance in poplar trees (Figure 2).

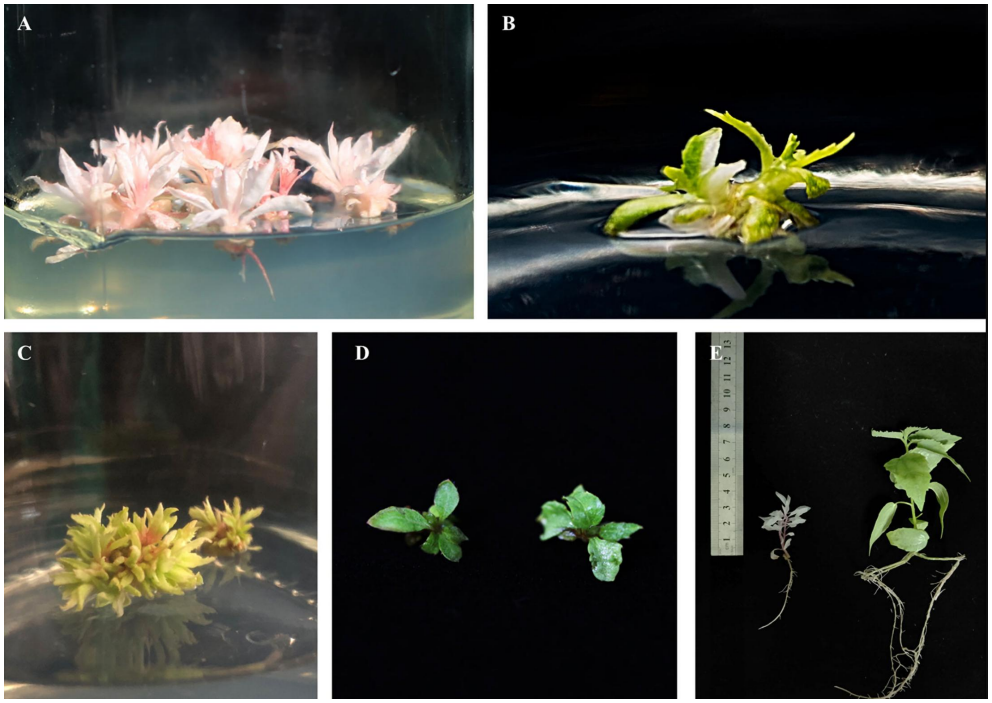

Figure 2 Representative Shanxin yang phenotypes resulting from *PDS* gene mutations (Adopted from Wang et al., 2020)
Image caption: Total albino (A), variegated (B), and pale green (C) phenotypes of the regenerated shoots. (D) Wild-type (WT) Shanxin yang shoots. (E) Comparison between the 1-month-old albino and WT seedlings (Adopted from Wang et al., 2020)

By analyzing Figure 2, it can be seen that poplar regeneration buds with PDS gene mutations exhibit varying

degrees of chlorosis. This indicates that CRISPR/Cas9-mediated gene editing is effective in poplar, and different sgRNA targets result in different mutation effects. This study provides important technical support for future improvements in the stress resistance traits of poplar.

Another example demonstrates that applying CRISPR/Cas9 to edit drought-resistant genes in poplar has yielded significant results. Compared to other programmable nucleases such as ZFNs and TALENs, the CRISPR/Cas9 system offers greater simplicity and efficiency. The research covers various plant-specific CRISPR/Cas9 vector systems, multiplex editing strategies, mutation analysis methods, and factors affecting editing efficiency (Figure 3). These case studies showcase the potential of CRISPR/Cas9 for precise and efficient gene editing in poplar, paving the way for the development of drought-resistant varieties.

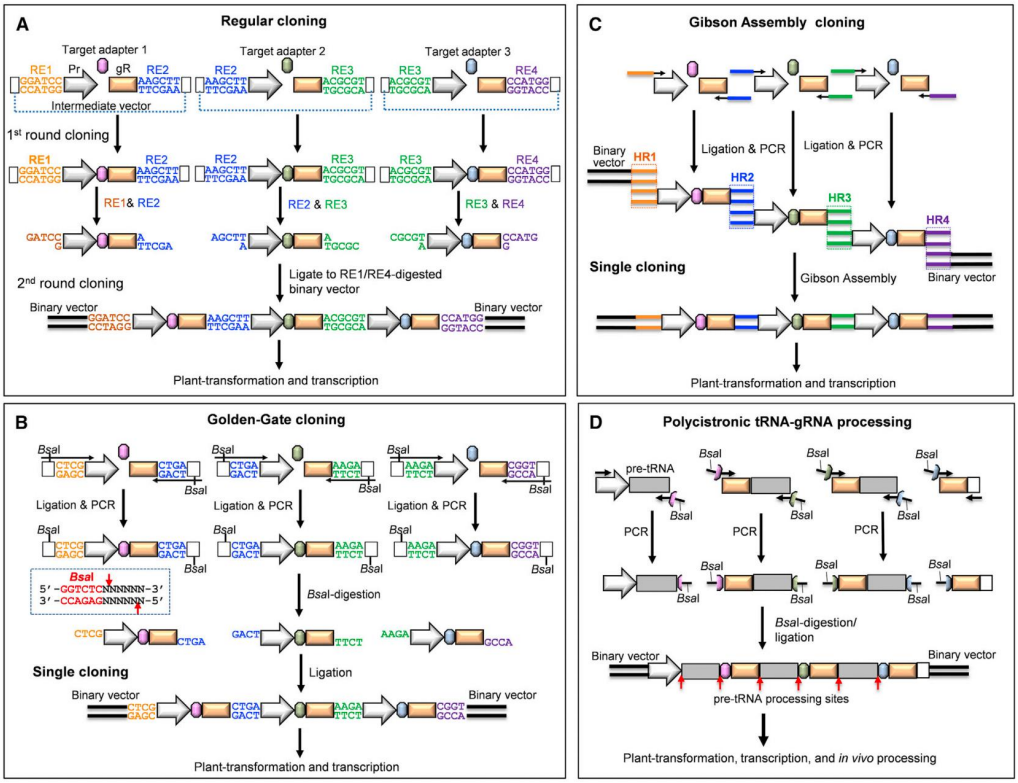

Figure 3 Strategies for generation of multiple sgRNA expression cassettes in a binary vector (Adopted from Ma et al., 2016)
Image caption: (A) Cloning of multiple sgRNA expression cassettes using multiple restriction enzymes (RE1–RE4). (B) Assembly of multiple (three as an example) sgRNA expression cassettes using Golden Gate ligation. (C) Cloning multiple (three as an example) sgRNA expression cassettes using Gibson assembly. (D) Generation of multiple sgRNAs by the polycistronic tRNA-gRNA gene (Adopted from Ma et al., 2016)

Figure 3 illustrates various strategies for generating multiple sgRNA expression cassettes in a binary vector. The figure details the implementation steps of four methods: multiple restriction enzyme cloning, Golden Gate assembly, Gibson assembly, and a polycistronic tRNA-gRNA system. These methods employ different technical approaches to connect multiple sgRNA expression cassettes, enabling multiplex genome targeting. These strategies allow for the simultaneous mutation of multiple genes or gene family members, which is crucial for studying complex traits and improving crop varieties. The application of these methods not only enhances the efficiency of gene editing but also broadens the scope of the CRISPR/Cas9 system in plant research.

5 Challenges and Considerations in CRISPR/Cas9-Mediated Gene Editing

5.1 Off-target effects and their mitigation

One of the primary challenges in CRISPR/Cas9-mediated gene editing is the occurrence of off-target effects, where the Cas9 enzyme cuts at unintended genomic locations. This can lead to unintended mutations, which may have deleterious effects on the plant's phenotype and overall health. Various strategies have been developed to

mitigate these off-target effects. For instance, the use of high-fidelity Cas9 variants and the optimization of guide RNA (gRNA) design can significantly reduce off-target activity (Arora and Narula, 2017; Badhan et al., 2021) Additionally, employing ribonucleoprotein (RNP) complexes instead of plasmid-based systems can enhance the specificity of the CRISPR/Cas9 system, as RNPs are rapidly degraded in the cell, reducing the window for off-target activity (Fan et al., 2015; Arora and Narula, 2017).

5.2 Regulatory and ethical considerations

The application of CRISPR/Cas9 technology in plants, including poplar, raises several regulatory and ethical considerations. Regulatory frameworks vary globally, with some countries adopting stringent regulations while others have more lenient policies. The primary concern is the potential for unintended ecological impacts and the ethical implications of genetic modifications. For instance, the release of genetically edited poplar trees into the environment could have unforeseen consequences on local ecosystems (Eş et al., 2019; Chen and Lu, 2020). Ethical considerations also include the potential for monopolization of CRISPR technology by a few entities, which could limit access for smaller research institutions and developing countries (Eş et al., 2019). Therefore, it is crucial to establish comprehensive regulatory guidelines that balance innovation with safety and ethical responsibility.

5.3 Environmental and ecological impacts

The environmental and ecological impacts of CRISPR/Cas9-mediated gene editing in poplar trees are significant considerations. While the technology holds promise for enhancing drought resistance, it is essential to evaluate the long-term ecological effects. For example, the introduction of drought-resistant poplar trees could alter local water cycles and affect other plant and animal species that depend on the same water resources (Fan et al., 2015; Chen and Lu, 2020). Additionally, there is a risk of gene flow from genetically edited poplars to wild relatives, which could lead to unintended ecological consequences (Eş et al., 2019). Therefore, thorough environmental impact assessments and long-term ecological studies are necessary to ensure that the benefits of CRISPR/Cas9-mediated gene editing do not come at the expense of environmental health.

6 Future Prospects and Research Directions

6.1 Innovations in CRISPR/Cas9 technology

The CRISPR/Cas9 system has undergone significant advancements since its inception, with new variants and methodologies continually emerging to enhance its precision, efficiency, and applicability. Recent innovations include the development of base editing and prime editing, which allow for more precise modifications without inducing double-strand breaks (Manghwar et al., 2019). Additionally, the use of CRISPR/Cas9 in combination with novel delivery systems, such as nanoparticle-based approaches, has shown promise in improving the efficiency of gene editing in plant species (Badhan et al., 2021). These advancements are crucial for the application of CRISPR/Cas9 in editing poplar drought resistance genes, as they can potentially increase the accuracy and effectiveness of the modifications.

6.2 Integrating CRISPR/Cas9 with other biotechnological approaches

Integrating CRISPR/Cas9 technology with other biotechnological approaches can further enhance its potential in developing drought-resistant poplar varieties. For instance, combining CRISPR/Cas9 with RNA interference (RNAi) or transcription activator-like effector nucleases (TALENs) can provide a multifaceted approach to gene regulation and editing (Bortesi and Fischer, 2015; Eş et al., 2019) Additionally, the use of multiplex CRISPR/Cas9 systems, which allow for the simultaneous targeting of multiple genes, can be particularly beneficial in addressing complex traits such as drought tolerance (Abdallah et al., 2022). This integration can lead to more robust and resilient poplar varieties by targeting multiple pathways involved in drought response.

6.3 Potential for commercial application and large-scale deployment

The commercial application and large-scale deployment of CRISPR/Cas9-edited poplar trees hold significant potential for the forestry industry. The ability to develop drought-resistant poplar varieties can lead to more sustainable forestry practices and improved biomass production under adverse environmental conditions (Chen and Lu, 2020). However, several challenges need to be addressed before large-scale deployment can be realized.

These include regulatory hurdles, public acceptance, and the need for comprehensive field trials to assess the long-term effects and stability of the edited traits (Ma et al., 2016; Eş et al., 2019). Despite these challenges, the successful commercial application of CRISPR/Cas9 technology in other crops provides a promising outlook for its use in poplar trees (Saber et al., 2019).

In conclusion, the future prospects of CRISPR/Cas9 technology in editing poplar drought resistance genes are promising, with ongoing innovations and integrations with other biotechnological approaches enhancing its potential. The pathway to commercial application and large-scale deployment, while challenging, is achievable with continued research and development.

7 Concluding Remarks

The application of CRISPR/Cas9 technology in editing poplar drought resistance genes has shown significant promise. This technology, characterized by its precision, efficiency, and versatility, has been successfully employed in various plant species to enhance traits such as drought tolerance, disease resistance, and overall crop improvement. Studies have demonstrated the potential of CRISPR/Cas9 to target specific genes associated with drought resistance, such as the *4CL* and *RVE7* genes in chickpea, which are crucial for lignin biosynthesis and circadian rhythm regulation, respectively. Additionally, the technology has been used to edit genes like *OsSAP* in rice, which plays a positive role in drought stress response. The multiplex CRISPR/Cas9 system has also been effective in generating wheat mutants with improved drought tolerance by targeting multiple TaSal1 loci.

The implications of these findings for poplar drought resistance improvement are profound. By leveraging CRISPR/Cas9 technology, researchers can precisely edit drought resistance genes in poplar, potentially leading to the development of more resilient poplar varieties. This could significantly enhance the sustainability and productivity of poplar plantations, especially in regions prone to drought. The ability to create targeted mutations and achieve stable, inheritable changes in the genome offers a powerful tool for accelerating breeding programs and developing poplar varieties with enhanced drought tolerance. Moreover, the use of CRISPR/Cas9 in poplar could pave the way for similar advancements in other forestry species, contributing to broader ecological and economic benefits.

The future of CRISPR/Cas9 in forestry looks promising, with the potential to revolutionize the way we approach genetic improvement in trees. As the technology continues to advance, it is expected that more precise and efficient gene editing methods, such as base editing and prime editing, will become available, further enhancing the capabilities of CRISPR/Cas93. Additionally, the development of novel delivery methods, including nanoparticle-based approaches, could improve the efficiency and applicability of CRISPR/Cas9 in forestry species. However, it is essential to address the ethical and regulatory challenges associated with the use of this technology to ensure its safe and responsible application. Overall, CRISPR/Cas9 holds great potential for advancing forestry research and developing trees that are better equipped to withstand environmental stresses, ultimately contributing to more sustainable and resilient forest ecosystems.

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Conflict of Interest Disclosure

The author affirms that this research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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